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Nabil A Rashdan[1], Alisia M Sim[2], Lin Cui[1], Kanchan Phadwal[1], Fiona L Roberts[1], Roderick Carter[3], Derya D Ozdemir[1], Peter Hohenstein[1], John Hung[3], Jakub Kaczynski[3], David E. Newby[3], Andrew H. Baker[3], Gerard Karsenty[4], Nicholas M Morton[3], Vicky E. MacRae[1]

1 The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, Edinburgh, EH25 9RG UK.

2 School of Chemistry, University of Edinburgh, Joseph Black Building, David Brewster Road, Edinburgh, EH9 3FJ, UK

3 Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, EH16 4TJ, UK

4 Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York 10032, USA.

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Abstract.

Arterial calcification is an important hallmark of cardiovascular disease and shares many similarities with skeletal mineralisation. The bone-specific protein osteocalcin (OCN) is an established marker of vascular smooth muscle cell (VSMC) osteochondrogenic trans-differentiation and a known regulator of glucose metabolism. However, the role of OCN in controlling arterial calcification is unclear. We hypothesised that OCN regulates calcification in VSMCs and sought to identify the underpinning signalling pathways. Immunohistochemistry revealed OCN co-localisation with VSMC calcification in human calcified carotid artery plaques. Additionally, 3 mM phosphate treatment stimulated OCN mRNA expression in cultured VSMCs (1.72 fold; p<0.001). Phosphate-induced calcification was blunted in VSMCs derived from OCN null mice (*Ocn*$^{-/-}$) compared to cells derived from Wild-Type (WT) mice (0.37 fold, p<0.001). *Ocn*$^{-/-}$ VSMCs showed reduced mRNA expression of the osteogenic marker *Runx2* (0.51 fold, p<0.01) and the sodium-dependent phosphate transporter, *PiT1* (0.70 fold, p<0.001), with an increase in the calcification inhibitor *Mgp* (1.42 fold, p<0.05) compared to WT. *Ocn*$^{-/-}$ VSMCs also showed reduced mRNA expression of *Axin2* (0.13 fold; p<0.001) and *Cyclin D* (0.71 fold; p<0.01), markers of Wnt signalling. CHIR99021 (GSK3β inhibitor) treatment increased calcium deposition in WT and *Ocn*$^{-/-}$ VSMCs (1 μM; p<0.001). *Ocn*$^{-/-}$ VSMCs however calcified less than WT cells (1 μM; 0.27 fold; p<0.001). *Ocn*$^{-/-}$ VSMCs showed reduced mRNA expression of *Glut1* (0.78 fold p<0.001), *Hex1* (0.77 fold p<0.01) and *Pdk4* (0.47 fold p<0.001). This was accompanied by reduced glucose uptake (0.38 fold, p<0.05). Subsequent mitochondrial function assessment revealed increased ATP-linked respiration (1.29 fold, p<0.05), spare respiratory capacity (1.59 fold, p<0.01) and maximal respiration (1.52 fold, p<0.001) in *Ocn*$^{-/-}$ versus WT VSMCs. Together these data suggest that OCN plays a crucial role in arterial calcification mediated by Wnt/β-catenin signalling through reduced maximal respiration. Mitochondrial dynamics may therefore represent a novel therapeutic target for clinical intervention.

Introduction.

The pathological process of arterial calcification shows many similarities with that of physiological bone development and formation (1). Crucially, vascular smooth muscle cells (VSMCs), the predominant cell type responsible for blood vessel calcification, can undergo phenotypic transition to chondrocytic, osteoblastic and osteocytic cells in a calcified environment (2, 3). Furthermore, phosphate accelerates this phenotypic trans-differentiation, evident in the loss of characteristic smooth muscle markers and the development of osteoblastic features, such as the expression of Runx2, Pit-1 and osteocalcin (OCN), and osteocyte markers including sclerostin and podopladin (Pdpn/E11) (2, 3). Arterial calcification also involves the reciprocal loss of recognised calcification suppressors, such as inorganic pyrophosphate (PP_i), matrix Gla protein (MGP) and fetuin A (4, 5). However, despite extensive investigations, the specific endocrine and molecular signalling mechanisms underpinning the arterial calcification process have yet to be fully defined.

Osteocalcin, the most abundant non-collagenous protein in bone(6), plays a key role in physiological and pathological calcification. Vitamin K-dependent post-translational modification of OCN involves the ɣ-carboxylation of three glutamic residues (Gla domain)(6), which confers OCN's hydroxyapatite binding ability. Intriguingly, recent findings have indicated that insulin signalling in osteoblasts favours bone resorption by osteoclasts, with decarboxylation of OCN occurring in resorption lacunae(7), resulting in increased circulating undercarboxylated OCN and a positive feedback loop on glucose metabolism via pancreatic insulin secretion(8, 9). Furthermore, OCN locally shifts VSMCs towards the glycolytic breakdown of glucose, and stimulates VSMC calcification *in vitro* (10). However, the cellular mechanisms through which OCN mediates this action, and the precise role of glucose metabolism in VSMC calcification remains to be fully elucidated. We have therefore undertaken analysis of clinical tissues in conjunction with *in vitro* studies in C57BL/6 and *Ocn*-null (*Ocn*$^{-/-}$) mice to investigate the functional role of OCN in glycolysis and arterial calcification.

Materials and Methods.

Human tissues.

Carotid artery plaque samples were obtained with appropriate ethical approval from patients undergoing carotid endarterectomy (Ethics Number: 15/ES/0094) and assessed subjectively for stability status. Research ethics committee approval (National Health Service Lothian Tissue Governance) and the written and informed consent of all participants were obtained. The samples were from three male patients, aged 54, 71, and 74. The first was a smoker with a history of chronic obstructive pulmonary hypertension. The second had a history of pancreatic cancer, hypertension, and hypercholesterolemia. The final patient had a history of myocardial infarction, pleural plaque, chronic obstructive pulmonary hypertension, severe left ventricular systolic dysfunction, and aortic stenosis. Human tissue was used in this study in conformation with the declaration of Helsinki.

Materials.

All materials unless stated otherwise were purchased from Sigma-Aldrich (Sigma, Dorset, UK).

Mice.

Ocn$^{-/-}$ mice were previously generated and genotyped as described (11). All animal experiments were performed under UK Home Office licensed approval in accordance with Directive 2010/63/EU of the European Parliament and were maintained in accordance with Home Office guidelines for the care and use of laboratory animals. C57BL/6 mice were supplied by Charles River Laboratories (Harlow, Essex, UK).

Primary murine VSMC isolation.

Primary aortic VSMCs were isolated from five-week old mice as described previously (3, 12, 13). Mice were euthanized by cervical dislocation. The aorta was then dissected, the adventitia removed, and the aorta cut open to expose the endothelial layer. After washing with Hanks' balanced salt solution (HBSS; Life Technologies, Paisley, UK), the aortae were cut longitudinally. Eight aortae were pooled together and incubated for 10 min at 37˚C in 1 mg/mL trypsin (Life Technologies, Paisley, UK) to remove any remaining endothelial cells Aortae were washed and incubated overnight at 37˚C in VSMC growth medium containing α-MEM, 10% foetal bovine serum and 1% gentamycin (Life Technologies, Paisley, UK) in a humidified 5% CO_2 incubator. Tissues were then washed and then incubated in 425 UI/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) for 4 hrs at 37˚C. The resulting cell suspension was centrifuged at 320g for 5 min. VSMC pellets were resuspended in culture medium and cultured for two passages in T25 tissue culture flasks (Corning Inc, Corning, NY) coated with 0.25 µg/cm^2 laminin (Sigma, Dorset, UK) to promote maintenance of the contractile differentiation state(14).

Induction of calcification.

VMSCs were seeded in growth medium at a density of 1 x 10^5 cells per well in 12 well plates (Corning Inc, Corning, NY). Calcification was induced as described previously (12, 15-17). In brief, cells were grown to confluence (day 0) and changed to calcification medium, which was prepared by supplementing growth medium with inorganic phosphate (P_i) to a final concentration of 3 mM. Cells were cultured in calcifying media for up to 7 days and media was changed every second/third day. All drugs and recombinant proteins were added at day 0.

Determination of calcification.

Calcium deposition was assessed as previously described (17, 18). Briefly, VSMCs were washed and incubated for 24 h in 0.6 N HCl at 4˚C. Free calcium was determined colorimetrically by a stable interaction with O-Cresolphthalein using a commercially available kit (Randox Laboratories Ltd., County Antrim, UK), and corrected for total protein concentration determined by DC assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Histology and immunohistochemistry.

Carotid artery plaques were dissected and fixed in 10% neutral buffered saline. Fixed tissues were dehydrated, embedded in paraffin wax and sectioned at 5 µm. Sections were de-waxed in xylene and stained with Von Kossa and nuclear fast red to visualise phosphate deposition or Alizarin red and counterstained with light green (Sigma, Dorset, UK) to assess calcium deposition. Sections were also stained with haematoxylin and eosin to assess tissue architecture. For Immunohistochemistry, sections were de-waxed in xylene followed by dehydration in graded alcohol. Antigen retrieval was accomplished using hot sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). Sections were then washed in Tris-Buffered Saline (TBS) and 0.025% Triton X-100 (wash buffer) (Sigma) and blocked in 5% normal goat serum and 1% BSA in TBS for 1 h. Following blocking slides were again washed and then incubated overnight at 4˚C with 4 µg IgG/mL anti-osteocalcin (sc-365797, Santa Cruz Biotechnology, Dallas, TX) in 1% BSA in TBS. Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in TBS for 15 min. The sections were then washed, incubated with diluted biotinylated mouse IgG kappa binding protein (sc-517142, Santa Cruz Biotechnology, Dallas, TX) (1:50 dilution) in 1% BSA in TBS for 30 min. Sections were then washed twice, then incubated for 30 min with VECTASTAIN® ABC Reagent (Vector Labs, Peterborough, UK). The sections were then incubated with DAB substrate reagent (0.06% DAB, 0.1% H2O2 in TBS) until the desired stain intensity developed. The sections were finally dehydrated, counterstained with haematoxylin and eosin and mounted in DePeX. Control sections were incubated with non-immune mouse IgG (4 µg IgG/mL) in place of the primary antibody.

Analysis of gene expression.

Total RNA was isolated using RNeasy mini columns (Qiagen, West Sussex, UK) following the manufacturer's directions. RNA was reverse transcribed and specific cDNAs were quantified

by real-time PCR using the SYBR green detection method as previously reported (19). Predesigned primers were purchased from Eurofins MWG Biotech (Ebersberg, Germany), Qiagen (Manchester, UK), Ambion (Cambridge UK) and Primer Design (Southampton, UK), with available sequences provided in the online Data Supplement (Suppl. Table S1).

Measurement of glucose uptake.

VSMCs were seeded at 10,000 cells per well in 96-well plates and incubated overnight. Cells were then cultured in calcifying medium for 48 h, followed by incubation in glucose-free DMEM (Life Technologies, Paisley, UK) for 1.5 hrs. VSMCs were then cultured with 5 mM fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Life Technologies, Paisley, UK) for 20 min. Cells were then washed twice with phosphate buffered saline (Life Technologies, Paisley, UK). Fluorescence was quantified at 485 nm excitation and 535 nm emission on a Synergy HT Multi Mode Microplate reader (BioTek, Swindon, UK) and corrected for total protein concentration (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Measurement of respiration.

VSMCs were seeded into XF24 V7 PS cell culture microplates (100777-004, Agilent Technologies LDA UK Limited) at ~20,000 cells per well. Cells were then cultured for 7 days with media changed every other day. On the day of respiratory analysis, cells were washed 3 times with Seahorse XF assay medium (102365-100, Agilent Technologies LDA UK Limited) supplemented with 10 mM Glucose and 2 mM pyruvate, pH adjusted with NaOH to 7.35 at 37°C. Cells were then incubated in a CO_2-free incubator at 37˚C for 30 min. Following this incubation microplates were placed in a Seahorse XF^{e}24 bioanalyser (Agilent Technologies LDA UK Limited). After basal measurements of oxygen consumption rate (OCR), respiratory ATP synthesis was inhibited by injecting oligomycin (an ATP synthase inhibitor) at a final concentration of 1 µM, causing a drop in OCR representative of ATP synthesis-linked respiration. Next the electron transport chain was uncoupled from ATP synthesis by injecting 1 µM final concentration of carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP), increasing OCR to a state of maximal respiration. Finally, respiration was abolished by injection of rotenone and antimycin A (inhibitors of mitochondrial complexes I and III respectively). at a concentration of 1 µM each. ATP-linked respiratory OCR was calculated from the difference between basal and oligomycin inhibited OCR. The difference between basal OCR and FCCP induced OCR was used to calculate the respiratory reserve capacity. The difference in OCR between FCCP and rotenone/antimycin A was considered maximal respiratory capacity. Proton leak respiratory OCR was calculated from the difference between oligomycin inhibited OCR and rotenone/antimycin A inhibited OCR.

Measurement of glycolysis.

Extracellular acidification rate (ECAR) was used to estimate the glycolytic rate. VSMCs were cultured as above on XF24 V7 PS cell culture microplates. VSMCs were washed 3 times with

XF assay medium (without glucose or pyruvate) and incubated in a CO_2-free incubator at 37˚C for 30 minutes. Glucose starved VSMCs were placed in a Seahorse XF^e24 bioanalyser. After basal measurements were taken, glucose was injected to a final concentration of 10 mM. The increase in ECAR following addition of glucose is representative of the cellular glycolytic flux. Oligomycin was then injected to a final concentration of 1 µM, inhibiting respiration derived ATP leading to an increase in ECAR. This increase was considered the Glycolytic reserve. At this point 2-deoxyglucose to a final concentration of 100 mM was injected to inhibit glycolysis. Glycolytic capacity was calculated from the difference between the oligomycin induced ECAR and the 2-deoxyglucose induced decrease.

Transfection assays.

VSMCs were transfected with either 60 pmol of mouse β-catenin (Santa Cruz Biotechnology, sc-29210) or scrambled control SiRNA (Santa Cruz Biotechnology, sc-37007) with siRNA transfection reagent (Santa Cruz Biotechnology, sc-29528), according to the manufacturer's instructions. The cells were harvested for experiments 72 hours post-transfection.

Immunoblotting.

Protein was extracted using radioimmunoprecipitation assay buffer supplemented with Halt Protease Inhibitor Cocktail EDTA-free (Thermo Scientific, 87785). Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23225) and samples were boiled with 2X sample buffer and then separated on a 4–12% Bis-Tris gel (Invitrogen). Proteins were then transferred to PVDF membranes (Immobilon-FL transfer membrane, IPFL00010) and blocked for 1 hour in LICOR blocking buffer. The membranes were incubated in primary antibodies (1: 250 mouse monoclonal anti-β-catenin, Santa Cruz Biotechnology, sc-7963, and 1:5000 Rabbit polyclonal anti-β-actin, GeneTex, GTX109639) in LICOR blocking buffer, at 4 °C overnight. Blots were then washed and incubated at first in goat anti-rabbit IRDye 680RD (926-68071) for 1 hour. Blots were then imaged using an Odyssey CLx Infrared Imaging System. The default settings used for blot image acquisition were resolution 169 µm, quality medium and laser intensities between 5–1.5. Blots were then incubated in goat anti-mouse IRDye 680 RD (926-68070) (Licor, Cambridge, UK) for 1 hour and reimaged.

Statistical analysis.

All data are presented as mean ± S.D. Data were analysed by two sample Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's range test, as appropriate. All statistical analysis was performed using Minitab 17 (Minitab Inc., Coventry, UK). $p<0.05$ was considered to be significant and p-values are represented as: *$p<0.05$; **$p<0.01$; ***$p<0.001$. n= individual cultures.

Results.

OCN is expressed in human calcified carotid artery plaque.

To investigate the role of OCN in arterial calcification, localisation studies were undertaken. Calcification of human carotid artery plaque was confirmed by Von Kossa (Fig. 1A (i & v)) and Alizarin red staining (Fig. 1A (ii & vi)). Immunohistochemistry confirmed OCN expression in calcified regions of the tissue (Fig. 1A (iii & vii)). Von Kossa (Fig. 1B (i & v)), Alizarin red (Fig. 1B (ii & vi)) and OCN (Fig. 1B (iii & vii)) staining was absent in non-calcified regions. Hence, OCN localisation in human atherosclerotic plaques correlates with *in situ* calcification.

Up-regulation of OCN during the VSMC calcification process.

Since arterial calcification is highly correlated with elevated serum phosphate levels (20), VSMCs were cultured in growth medium containing 3 mM P_i. Three mM P_i induced an increase in VSMCs calcium deposition (determined by HCl leaching) by day 7 compared to cells cultured in control medium (2.26 fold, $p<0.001$; Fig. 1C). This was accompanied by an increase in *Ocn* mRNA expression (1.72 fold; $p<0.001$ Fig. 1D). Consistent with previous studies (16, 17), 3 mM P_i induced osteogenic transition of VSMCs, as demonstrated by up-regulated expression of *Runx2* (1.95-fold; $p<0.01$); and *Pit-1* (7.18-fold; $p<0.001$) (Fig. 1E) while the calcification inhibitor *Mgp* (0.66-fold; $p<0.01$) was reduced by 3 mM P_i (Fig. 1E). These data suggest that *Ocn* expression of is positively regulated by elevated P_i.

OCN ablation attenuates P_i-induced VSMC calcification.

To establish the functional role of OCN in arterial calcification, further studies were undertaken using VSMCs derived from *Ocn*$^{-/-}$ mice. In the presence of 3 mM P_i medium for 7 days, a reduction in calcium deposition was observed in *Ocn*$^{-/-}$ VSMCs compared to Wild Type (WT) control cells, as determined by HCl leaching (0.37 fold, $p<0.001$; Fig. 1F). Consistent with these data, *Ocn*$^{-/-}$ VSMCs showed a reduction in the mRNA expression of *Runx2* (0.51 fold, $p<0.01$), and *Pit-1* (0.70 fold, $p<0.001$ (Fig. 1G), with a concomitant increase in the calcification inhibitor *Mgp* compared to WT cells (1.42 fold, $p<0.05$; Fig. 1G). This observation suggests a direct link between *Ocn* expression and VSMC calcification.

OCN ablation diminishes Wnt signalling in calcifying VSMCs.

The Wnt signalling pathway plays an established role in osteogenesis and can impact on arterial calcification (21, 22). In the present study, calcifying VSMCs showed increased mRNA expression of the Wnt targets *Axin2* (2.84 fold; $p<0.01$), *Cyclin d* (1.87 fold; $p<0.05$) and dickkopf-related protein 1 (*Dkk1*; 2.61 fold; $p<0.05$) (Fig. 2A). Functionally, CHIR99021, a glycogen synthase kinase 3β (GSK3β) inhibitor and Wnt signalling activator, accelerated P_i-induced VSMC calcium deposition from 1 nM (4.18 fold, $p<0.01$, Fig. 2B). Conversely, exogenous recombinant DKK1 (a Wnt signalling pathway inhibitor) reduced P_i-induced calcium deposition in VSMCs from a minimum concentration of 2.5 ng/mL (0.42 fold $p<0.05$;

Fig. 2C). Subsequent studies investigated whether alterations in Wnt signalling underpinned the pro-calcification role of OCN. *Ocn*$^{-/-}$ VSMCs showed reduced mRNA expression of Wnt signalling markers *Axin2* (0.13 fold; p<0.001), *Cyclin d* (0.71 fold; p<0.01; Fig. 2D). Conversely, *Dkk1* expression was increased (1.55 fold, p<0.05, Fig. 2D). Treatment with CHIR99021 increased calcium deposition in both WT and *Ocn*$^{-/-}$ VSMCs (1 µM; p<0.001; Fig. 2E). However, CHIR99021-induced calcification was blunted in *Ocn*$^{-/-}$ VSMCs compared to WT (1 µM; 0.27 fold; p<0.001).

Glucose metabolism regulated by OCN in calcifying VSMCs.

Accumulating evidence suggests that aerobic glycolysis may be a characteristic feature of VSMCs osteogenic transdifferentiaton during the calcification process (23). We therefore determined the effects of VSMCs calcification on glucose uptake using the fluorescent glucose analogue 2-NBDG. Normalised fluorescence was increased by culture of VSMCs with 3 mM P_i for 7 days (1.98 fold, p<0.05) compared to control cells (Fig. 3A). Consistent with this finding, calcifying VSMCs showed increased mRNA expression of *Glut1* (1.98 fold, p<0.01), the rate limiting enzyme of glucose metabolism *Hex1* (2.65 fold, p<0.01) and *Pdk4* (2.17 fold p<0.01) (Fig. 3B).

Together, these data suggest increased glucose metabolism during P_i-induced VSMCs calcification. Therefore, further studies interrogating the consequences of altered glucose metabolism were undertaken using pharmacological inhibitors. Interestingly, the inhibition of glucose uptake with fasentin, a GLUT1 specific inhibitor, accelerated P_i-induced VSMC calcification (20uM, 72%, p<0.01; Fig. 3C). Similarly, the hexokinase inhibitor 2-deoxyglucose (2-DG), increased calcium deposition (100 µM; 97% increase, p<0.05; Fig. 3D). Dichloroacetate (DCA), a general pyruvate dehydrogenase kinase (PDK) inhibitor which increases pyruvate dehydrogenase PDH activity, decreased P_i-induced VSMCs calcification (1mM; 50%, p<0.05; Fig. 3E).

A Seahorse XF^{e}24 flux bioanalyser was used to evaluate glycolysis (Fig. 3G) as well as oxidative phosphorylation (Fig. 3H) in VSMCs cultured with or without 3 mM P_i. Three mM P_i induced a decrease in both glycolytic capacity (0.37 fold, p<0.001) and reserve (0.74 fold, p<0.05) (Fig. 3I). Calcifying conditions also decreased ATP-linked respiration (0.58 fold p<0.01), spare respiratory capacity (0.37 fold, p<0.05) and maximal respiration (0.49 fold, p<0.01) (Fig. 3J). Consistent with the critical key role of OCN in glucose uptake and utilisation(10), *Ocn*$^{-/-}$ VSMCs exhibited reduced 2-NBDG fluorescence compared to WT cells (0.38 fold, p<0.05, Fig. 4A). This decrease in glucose uptake was accompanied by a marked reduction in the mRNA expression of *Glut1* (0.78 fold p<0.001), *Hex1* (0.77 fold p<0.01) and *Pdk4* (0.47 fold p<0.001) in calcifying *Ocn*$^{-/-}$ VSMCs compared to WT control cells (Fig. 4B). *Ocn*$^{-/-}$ VSMCs had higher rates of glycolysis (Fig. 4C) and oxidative phosphorylation (Fig. 4D) compared to WT VSMCs, indicative of increased energy demand. *Ocn*$^{-/-}$ VSMCs also had higher basal glycolysis (2.85 fold, p<0.05), glycolytic capacity (1.58 fold, p<0.001) as well as glycolytic reserve (1.43 fold, p<0.001) (Fig. 4E) compared to WT cells. ATP-linked respiration

(1.29 fold, p<0.05), spare respiratory capacity (1.59 fold, p<0.01) and maximal respiration (1.52 fold, p<0.001) (Fig. 4F) were increased in *Ocn*$^{-/-}$ versus WT VSMCs. *β-Catenin* knockdown (Fig. 5A) induced a significant decrease (0.42 fold, p<0.01) in β-catenin protein expression (Fig. 5B). *β-Catenin* siRNA reduced the expression of the Wnt signalling markers *Axin2* (0.78 fold; p<0.05), *Cyclin d* (0.73 fold; p<0.001; Fig. 5C). Comparable to osteocalcin ablation in VSMCs, *β-catenin* knockdown also induced a significant increase in glycolytic capacity (1.90 fold, p<0.05) as well as glycolytic reserve (1.42 fold, p<0.05) (Fig. 5C).

Discussion.

The osteoblast-specific protein OCN mediates crucial inter-organ communications. Indeed, the inactivation of OCN secreted in the bone ECM influences an extensive range of physiological processes outwith bone, including fat accumulation, skeletal muscle function, cognition and reproduction(24). This investigation addressed the hypothesis that OCN induces arterial calcification through mechanisms underpinned by decreased glycolysis. Our data have shown that the expression of *Ocn* is associated with arterial calcification *ex vivo* and *in vitro*. Moreover, we have shown that OCN may be directly involved in Wnt-driven VSMC calcification via the regulation of mitochondrial activity. Our work offers new insight into the role of OCN in cardiovascular disease and provides direct evidence to suggest that OCN contributes to the pathological process of arterial calcification.

Our *in vitro* investigations revealed a notable up-regulation of *Ocn* with phosphate-induced VSMC calcification. Furthermore, a concomitant increase in the expression of *Runx2*, a recognized regulator of osteoblastic differentiation and matrix calcification of VSMCs, was observed. Indeed, there is a substantial body of evidence linking RUNX2 elevation with arterial calcification. RUNX2 has been detected in calcified atherosclerotic lesions in people (25-28) and is up-regulated in VSMCs undergoing transdifferentiation to osteochondrogenic cells in mouse models of arterial intimal calcification (29) and medial calcification (30). Additionally, calcified VSMCs showed up-regulated expression of the sodium-dependent phosphate co-transporter PiT-1. Increased PiT-1 expression leads to elevated intracellular phosphate and induces the osteogenic conversion of VSMCs (31). A parallel decrease in matrix Gla protein, an established inhibitor of arterial calcification (5, 32-36) was observed.

Consistent with these findings, we report an association between OCN expression and calcified carotid plaques in patients. These data confirm and extend previous studies showing increased OCN expression in calcified carotid plaques (37, 38) and arterial lesions as they progress from fatty streaks to fibro-calcific plaques (39). Furthermore, previous clinical analyses have revealed circulating uncarboxylated OCN to be positively associated with coronary artery calcification (40).

The utilization of the novel *Ocn* knockout mouse model was central to this investigation. The employment of VSMCs derived from this mouse line emphatically highlights a functional role for OCN in phosphate-induced arterial calcification. Additionally, the reduced expression of

both *Runx2* and *PiT-1* in *Ocn*$^{-/-}$ VSMCs reveals that OCN may regulate the osteogenic differentiation of VSMCs. Furthermore, our data suggest that OCN may mediate VSMC calcification through increased expression of the calcification inhibitor MGP.

Mechanistically our data suggests that OCN induces calcification through the Wnt/β-catenin signalling pathway, corroborating and extending previous studies highlighting Wnt signaling as a key regulator of arterial calcification. Paracrine Wnt signals are activated via bone morphogenetic protein-2–induced Msx2 expression within calcified plaques in LDLR$^{-/-}$ mice, resulting in increased osteogenesis (41). Additional studies have shown that LDL receptor–related protein 5 (LRP5) expression is upregulated at sites of osteogenesis in human degenerative valves (42) and that atorvastatin inhibits hypercholesterolemia-induced calcification of rabbit aortic valves by downregulating LRP5 and β-catenin levels (43). This study is the first to highlight the Wnt/β-catenin signalling pathway as a hub in driving OCN-regulated arterial calcification.

A potential link between OCN and glucose metabolism in calcifying smooth muscle cells has recently been suggested (10). This is consistent with recent seminal research showing OCN to be a key metabolic hormone inducing increased insulin production and sensitivity (8, 9). *Ocn*$^{-/-}$ VSMCs showed higher basal glycolysis and maximal respiration rates compared to WT VSMCs, which is indicative of these cells having greater energy demand, and increased ATP-linked respiration. This in turn may inhibit calcification in an Ectonucleotide Pyrophosphatase/Phosphodiesterase 1(ENPP1)-dependent manner (44), whereby ENPP1 converts ATP to pyrophosphate, a potent calcification inhibitor (45). Further studies employing pyrophosphate-deficient mouse models (e.g. *Enpp1*$^{-/-}$ and *Abcc6*$^{-/-}$ mice) are required to more fully investigate the role of pyrophosphate biogenesis as a key component of mitochondrial contributions to calcification downstream of this newly elucidated OCN /Wnt axis.

In summary, we demonstrate a functional role for OCN in the pathological process of arterial calcification. Mechanistically, this role may be mediated by Wnt signalling and be dependent on decreased maximal respiration. To the best of our knowledge, this is the first study demonstrating an interaction between Wnt signalling and cellular metabolism within the context of VSMC calcification, highlighting new potential therapeutic strategies for the inhibition of arterial calcification.

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References.

1. Zhu D, Mackenzie NCW, Farquharson C, Macrae VE. Mechanisms and clinical consequences of vascular calcification. Frontiers in Endocrinology. 2012;3.
2. Speer MY, Yang H-Y, Brabb T, Leaf E, Look A, Lin W-L, et al. Smooth Muscle Cells Give Rise to Osteochondrogenic Precursors and Chondrocytes in Calcifying Arteries. Circulation Research. 2009;104(6):733.
3. Zhu D, Mackenzie NCW, Millán JL, Farquharson C, MacRae VE. The Appearance and Modulation of Osteocyte Marker Expression during Calcification of Vascular Smooth Muscle Cells. PLoS ONE. 2011;6(5):e19595.
4. Rutsch F, Ruf N, Vaingankar S, Toliat MR, Suk A, Hohne W, et al. Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. Nat Genet. 2003;34(4):379-81.
5. Murshed M, Harmey D, Millan JL, McKee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. Genes Dev. 2005;19(9):1093-104.
6. Hauschka PV, Lian JB, Cole DEC, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev. 1989;69:990-1047.
7. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, et al. Insulin Signaling in Osteoblasts Integrates Bone Remodeling and Energy Metabolism. Cell. 2010;142(2):296-308.
8. Ferron M, Lacombe J. Regulation of energy metabolism by the skeleton: osteocalcin and beyond. Archives of biochemistry and biophysics. 2014;561:137-46.
9. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. Cell. 2010;142(2):309-19.
10. Idelevich A, Rais Y, Monsonego-Ornan E. Bone Gla protein increases HIF-1alpha-dependent glucose metabolism and induces cartilage and vascular calcification. Arterioscler Thromb Vasc Biol. 2011;31(9):e55-71.
11. Ducy P. Increased bone formation in osteocalcin-deficient mice. Nature. 1996;382:448-52.
12. Zhu D, Hadoke PWF, Wu J, Vesey AT, Lerman DA, Dweck MR, et al. Ablation of the androgen receptor from vascular smooth muscle cells demonstrates a role for testosterone in vascular calcification. 2016;6:24807.
13. Zhu D, Rashdan NA, Chapman KE, Hadoke PW, MacRae VE. A novel role for the mineralocorticoid receptor in glucocorticoid driven vascular calcification. Vascul Pharmacol. 2016;86:87-93.
14. Johnson KA, Polewski M, Terkeltaub RA. Transglutaminase 2 Is Central to Induction of the Arterial Calcification Program by Smooth Muscle Cells. Circulation Research. 2008;102(5):529-37.
15. Zhu D, Mackenzie NC, Farquharson C, Macrae VE. Mechanisms and clinical consequences of vascular calcification. Front Endocrinol (Lausanne). 2012;3:95.
16. Zhu D, Mackenzie NCW, Shanahan CM, Shroff RC, Farquharson C, MacRae VE. BMP-9 regulates the osteoblastic differentiation and calcification of vascular smooth muscle cells through an ALK1 mediated pathway. Journal of Cellular and Molecular Medicine. 2015;19(1):165-74.
17. Zhu D, Mackenzie NC, Millan JL, Farquharson C, Macrae VE. Upregulation of IGF2 expression during vascular calcification. Journal of molecular endocrinology. 2014;52(2):77-85.
18. Mackenzie NC, Zhu D, Longley L, Patterson CS, Kommareddy S, MacRae VE. MOVAS-1 cell line: a new in vitro model of vascular calcification. International journal of molecular medicine. 2011;27(5):663-8.

19. Huesa C, Zhu D, Glover JD, Ferron M, Karsenty G, Milne EM, et al. Deficiency of the bone mineralization inhibitor NPP1 protects against obesity and diabetes. Disease Models & Mechanisms. 2014;7(12):1341-50.
20. Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. Circ Res. 2001;89(12):1147-54.
21. Freise C, Kretzschmar N, Querfeld U. Wnt signaling contributes to vascular calcification by induction of matrix metalloproteinases. BMC cardiovascular disorders. 2016;16(1):185.
22. Montes de Oca A, Guerrero F, Martinez-Moreno JM, Madueno JA, Herencia C, Peralta A, et al. Magnesium inhibits Wnt/beta-catenin activity and reverses the osteogenic transformation of vascular smooth muscle cells. PLoS One. 2014;9(2):e89525.
23. Leem J, Lee I-K. Mechanisms of Vascular Calcification: The Pivotal Role of Pyruvate Dehydrogenase Kinase 4. Endocrinology and Metabolism. 2016;31(1):52-61.
24. Wei J, Karsenty G. An overview of the metabolic functions of osteocalcin. Current osteoporosis reports. 2015;13(3):180-5.
25. Moe SM, Duan D, Doehle BP, O'Neill KD, Chen NX. Uremia induces the osteoblast differentiation factor Cbfa1 in human blood vessels. Kidney Int. 2003;63(3):1003-11.
26. Tyson KL, Reynolds JL, McNair R, Zhang Q, Weissberg PL, Shanahan CM. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. Arterioscler Thromb Vasc Biol. 2003;23(3):489-94.
27. Aikawa E, Nahrendorf M, Sosnovik D, Lok VM, Jaffer FA, Aikawa M, et al. Multimodality molecular imaging identifies proteolytic and osteogenic activities in early aortic valve disease. Circulation. 2007;115(3):377-86.
28. Bobryshev YV. Transdifferentiation of smooth muscle cells into chondrocytes in atherosclerotic arteries in situ: implications for diffuse intimal calcification. J Pathol. 2005;205(5):641-50.
29. Naik V, Leaf EM, Hu JH, Yang HY, Nguyen NB, Giachelli CM, et al. Sources of cells that contribute to atherosclerotic intimal calcification: an in vivo genetic fate mapping study. Cardiovasc Res. 2012;94(3):545-54.
30. Lin ME, Chen T, Leaf EM, Speer MY, Giachelli CM. Runx2 Expression in Smooth Muscle Cells Is Required for Arterial Medial Calcification in Mice. Am J Pathol. 2015;185(7):1958-69.
31. Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res. 2006;98(7):905-12.
32. Murshed M, Schinke T, McKee MD, Karsenty G. Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. J Cell Biol. 2004;165(5):625-30.
33. Kaartinen MT, Murshed M, Karsenty G, McKee MD. Osteopontin upregulation and polymerization by transglutaminase 2 in calcified arteries of Matrix Gla protein-deficient mice. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2007;55(4):375-86.
34. Marulanda J, Gao C, Roman H, Henderson JE, Murshed M. Prevention of arterial calcification corrects the low bone mass phenotype in MGP-deficient mice. Bone. 2013;57(2):499-508.
35. Khavandgar Z, Roman H, Li J, Lee S, Vali H, Brinckmann J, et al. Elastin haploinsufficiency impedes the progression of arterial calcification in MGP-deficient mice. J Bone Miner Res. 2014;29(2):327-37.

36. Khavandgar Z, Murshed M. Sphingolipid metabolism and its role in the skeletal tissues. Cellular and Molecular Life Sciences. 2015;72(5):959-69.
37. Foresta C, Strapazzon G, De Toni L, Fabris F, Grego F, Gerosa G, et al. Platelets express and release osteocalcin and co-localize in human calcified atherosclerotic plaques. Journal of thrombosis and haemostasis : JTH. 2013;11(2):357-65.
38. Bini A, Mann KG, Kudryk BJ, Schoen FJ. Noncollagenous bone matrix proteins, calcification, and thrombosis in carotid artery atherosclerosis. Arterioscler Thromb Vasc Biol. 1999;19(8):1852-61.
39. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol. 2001;21(12):1998-2003.
40. Choi BH, Joo NS, Kim MJ, Kim KM, Park KC, Kim YS. Coronary artery calcification is associated with high serum concentration of undercarboxylated osteocalcin in asymptomatic Korean men. Clinical endocrinology. 2015;83(3):320-6.
41. Shao J-S, Cheng S-L, Pingsterhaus JM, Charlton-Kachigian N, Loewy AP, Towler DA. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. Journal of Clinical Investigation. 2005;115(5):1210-20.
42. Caira FC, Stock SR, Gleason TG, McGee EC, Huang J, Bonow RO, et al. Human degenerative valve disease is associated with up-regulation of low-density lipoprotein receptor-related protein 5 receptor-mediated bone formation. J Am Coll Cardiol. 2006;47(8):1707-12.
43. Rajamannan NM, Nealis TB, Subramaniam M, Pandya S, Stock SR, Ignatiev CI, et al. Calcified rheumatic valve neoangiogenesis is associated with vascular endothelial growth factor expression and osteoblast-like bone formation. Circulation. 2005;111(24):3296-301.
44. Patel JJ, Zhu D, Opdebeeck B, D'Haese P, Millán JL, Bourne LE, et al. Inhibition of arterial medial calcification and bone mineralization by extracellular nucleotides: The same functional effect mediated by different cellular mechanisms. Journal of Cellular Physiology. 2018;233(4):3230-43.
45. Mackenzie NCW, Zhu D, Milne EM, van 't Hof R, Martin A, Quarles DL, et al. Altered Bone Development and an Increase in FGF-23 Expression in <italic>Enpp1^{−/−}</italic> Mice. PLoS ONE. 2012;7(2):e32177.

Figure legends.

Figure 1. Osteocalcin is expressed in calcified human cardiovascular tissue and plays a functional role in VSMCs calcification.

A) Calcification of human carotid artery plaque was confirmed by (i & v) Von Kossa (arrows indicate positive Von Kossa staining) and (ii & vi) Alizarin red staining (arrows indicate positive Alizarin red staining). (iii & vii) OCN expression was observed in the plaque around the area of calcification (arrows indicate positive OCN staining) (iv and viii) IgG control. B) Non-calcified human carotid artery plaque was negative for (i & v) Von Kossa, (ii & vi) Alizarin red staining. (iii & vii) OCN expression (iv and viii) shows IgG control. VSMCs were cultured with 3 mM P_i or control (1 mM P_i) for 7 days. C) VSMCs calcium deposition (determined by HCL leaching) in cells cultured in 3 mM P_i compared to cells cultured in control medium. D) *Ocn* mRNA expression E) 3 mM Pi induced the expression of osteogenic markers *Runx2*, *Pit-1*, while, *Mgp* was significantly reduced by 3 mM P_i. F) Calcium content (µg/mg protein) by HCl leaching in $Ocn^{-/-}$ (white bar) and WT (filled bar) VSMCs were cultured with control (1 mM Pi) or high

phosphate (3 mM P_i) medium for 7 days. G) Relative change in osteogenic marker mRNA expression of *Ocn*$^{-/-}$ cells compared to WT cells; *Runx2 Pit1*, and *Mgp*. Scale bars= 50 µm. Results are presented as mean ±S.D. *p* vs. control, (n=6).

Figure 2. Wnt signalling contributes to VSMCs calcification and is decreased in calcifying *Ocn*$^{-/-}$ VSMCs.

VSMCs were cultured with control 1 mM P_i or 3 mM P_i medium for 7 days. A) Fold change in the mRNA expression of Wnt signalling targets *Axin2*, *Cyclin d*, and *Dkk1*. Calcium content (µg/mg protein) was quantified in VSMCs cultured in 3 mM P_i medium in the presence/absence of B) CHIR99021 (Wnt activator) (0.001-10 µM) and C) DKK1(Wnt inhibitor) from 2.5 - 10 ng/ml. D) Fold change in the mRNA expression of *Axin2*, *Cyclin d Dkk1* in *Ocn*$^{-/-}$ (square) and WT (circle) VSMCs after 7 days in 3 mM P_i medium. E) Calcium content (µg/mg protein) was quantified in *Ocn*$^{-/-}$ and WT VSMCs cultured in 3 mM P_i medium in the presence/absence of CHIR99021 0.1 – 10 µM. Results are presented as mean ±S.D. *p* vs. WT, (n=6).

Figure 3. Glucose uptake and metabolism in calcifying VSMCs.

VSMCs were cultured with control or 3 mM P_i medium. A) Normalised fluorescence of the glucose analog 2-NBDG (fl/mg protein) determined after 48 hrs in culture (n=6). Fold change in the mRNA expression of glucose metabolism genes determined after 7 days in culture, B) *Glut1, Hex1,* and *Pdk4* (n=6). Calcium content (µg/mg protein) was quantified in the presence/absence of: C) Fasentin (20-60 µM) induced calcification (n=6). D) 2-Deoxyglucose (1-100 µM) increased calcium content at 100 µM (n=6). E) DCA (0.1-10 mM) induced a significant reduction in calcium content from 1 mM (n=6). G) Extracellular acidification rate (ECAR) in control (white squares) and 3 mM P_i (black diamonds) cultured VSMCs during a glycolysis stress test (n=5). H) Oxygen consumption rate (OCR) in control (white squares) and 3 mM P_i (black diamonds) cultured VSMCs during a mitochondrial stress test (n=5). I) Glycolytic capacity and reserve were significantly decreased by 3 mM P_i (squares) compared to control (circles) (n=5). J) ATP-linked respiration, spare respiratory capacity and maximal respiration were also significantly decreased by 3 mM P_i (squares) compared to control (circles). Results are presented as mean ±S.D. *p* vs. control.

Figure 4. Altered glucose metabolism in calcifying *Ocn*$^{-/-}$ VSMCs.

Ocn$^{-/-}$ and WT VSMCs were cultured with control or 3 mM P_i medium for up to 7 days. A) Normalised fluorescence of the glucose analog 2-NBDG (fl/mg protein) (n=8). B) Fold change in the mRNA expression of glucose metabolism genes in WT (circle) and *Ocn*$^{-/-}$ (square) *Glut1, Hex1,* and *Pdk4* (n=6). C) ECAR during glycolysis stress test for WT (white squares) and *Ocn*$^{-/-}$ (black diamonds) VSMCs D) OCR during mitochondrial stress test for WT (white squares) and *Ocn*$^{-/-}$ (black diamonds) VSMCs (n=6). E) Basal glycolysis, glycolytic capacity and

glycolytic reserve were significantly higher in *Ocn*$^{-/-}$ VSMCs (square) compared to WT (circle) (n=5). F) ATP-linked respiration, spare respiratory capacity and maximal respiration were also significantly higher in *Ocn*$^{-/-}$ VSMCs (square) compared to WT (circle) (n=5). Results are presented as mean ±S.D. *p* vs. WT.

Figure 5. Altered glucose metabolism by *β-catenin* knockdown in VSMCs.

WT VSMCs were transfected with either scrambled or β-catenin siRNA. A) After 72h cellular β-catenin protein content was determined by western blot. B) Fold change in β-catenin protein compared to scrambled siRNA control (n=3). C) Fold change in the mRNA expression of *Axin2*, *Cyclin d* in β-catenin compared to scrambled siRNA treated VSMCs (n=5). C) ECAR during glycolysis stress test for scrambled (white squares) and β-catenin (black diamonds) siRNA treated VSMCs (n=9). D) Glycolytic capacity and glycolytic reserve were significantly higher in in β-catenin (square) compared to scrambled (circle) siRNA treated VSMCs (n=9). Results are presented as mean ±S.D. *p* vs. scrambled siRNA.